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The evaluation of the antiviral effects of aqueous extracts of red and yellow onions (Allium Cepa) against avian influenza virus subtype H9N2

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ABSTRACT

Avian influenza virus subtype H9N2 causes important economic losses in industrial poultry worldwide. Biosecurity and vaccination have not completely prevented the outbreak of avian influenza virus subtype H9N2 in poultry, and there are no appropriate medicines to treatment it. Onion is one of the plants used from the ancient times both as food and medicine. The purpose of this study was to evaluate the antiviral effects of aqueous extract of red and yellow onion against avian influenza virus subtype H9N2. First, a study was done to evaluate the toxic effects of the extracts on the embryonated chicken eggs. For antiviral evaluation, three mixtures prepared the mixture of the virus and red onion extract, the mixture of the virus and yellow onion extract, and the mixture of the virus and PBS, as a control group. The mixtures were separately inoculated to the chorioallantoic sac of the embryonated eggs after 2, 8 and 24 hours incubation at room temperature. Mortality rate and hemagglutination assay titers were recorded for the evaluation. The results indicated that the red onion extract decreases mortality of the embryos and the yellow onion extract increases the life of the embryos, and both of the extracts decrease HA titers. In conclusion, it seems that both extracts especially aqueous extract of red onion not only destroys the avian influenza virus subtype H9N2, but also they probably decrease the propagation of the virus in embryonated chicken eggs.

Keywords

Avian Influenza, H9N2, Onion, Aqueous extract, Antivirus

Abbreviations

HA: Hemagglutination
AI: Avian Influenza
CAF: Chorioallantoic Fluid

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Introduction

Avian influenza (AI) virus subtype H9N2 causes important economic losses in industrial poultry worlwide [1]. Subtype H9N2 which is a low pathogenic avian influenza virus was first isolated and identified in the 1960s [2]. This subtype rapidly spread in industrial poultry of Iran after the first report in 1998 [3]. Biosecurity and vaccination have not completely prevented the outbreak of AI virus subtype H9N2 in poultry and at the present, there is no appropriate anti-influenza drug for treatment of infected food animals such as commercial poultry [4].

Common onions (Allium Cepa) are perennials plants which are cultivated throughout the world [5]. These plants are used medicinally as well as for food [5]. Onions have been used in folk medicine for thousands of years [1]; furthermore, different studies have revealed that onions have several therapeutic properties such as antimicrobial activity [6], antiparasitic [7], antiviral [8], antifungal [2], antioxidant and anti-inflammatory activities [5]. Limited available data exhibit inhibitory effects of onion against human immunodeficiency virus (HIV), herpes simplex virus type 1, poliovirus type 1, Para-influenza virus type 3, and potato virus [8, 9, 10].

As no data available regarding the antiviral effect of onion against avian influenza virus subtype H9N2, the purpose of this study was to evaluate antiviral effects of aqueous extract of onion against the virus. Red and yellow onions have had different antibacterial effects [11], therefore, in this study anti-influenza effect of the two types of onion was also compared.

Results

Cytotoxicity of the aqueous extracts of the red and yellow onions. Diluted and even undiluted aqueous red and yellow onion extracts had no adverse effect on embryo's viability. No mortality rates were recorded among embryos.

Mortality rate

Mortality rate of embryos was as same as in eggs that inoculated the mixture of the AI virus and aqueous extract of red or yellow onion after 2, and 8 hours incubation at room temperature; The two mixtures that incubated 2 hours, caused death of 60% and 40% of the embryos on the second and third day post inoculation respectively. Although the mortality rate of the control group on the second day post inoculation was 100%, statistically there was no significant difference between control group and treatment groups (P= 0.30). The two other mixtures that incubated 8 hours caused the death of 80% and 20% on the second and third day post inoculation respectively. While the Mortality rate of the control group was 100% on second day post inoculation. The statistical comparison of three groups, two treatment groups, and one control group, showed that there is no significant difference between them (p= 0.62), (table 2 and 3).

Table 2
Mortality rate of embryos in eggs after day 2, and 3 of inoculation of the mixture of the AI virus and aqueous extract of red or yellow onion or PBS, after 2 hours incubation at room temperature.

Groups	Mortality rate (%)	
	Day 3	Day 2
A	60 ± 24.5	40
B	60 ± 24.5	40
C	100	0
P value	0.300	NA*

*The data were not analyzed
Group A, received the mixture of the virus and the red onion extract.
Group B, received the mixture of the virus and the yellow onion extract.
Group C, received the mixture of the virus and the PBS.

Table 3
Table 3: Mortality rate of embryos in eggs after day 2, and 3 of inoculation of the mixture of the AI virus and aqueous extract of red or yellow onion or PBS, after 8 hours incubation at room temperature.

Groups	Mortality rate (%)	
	Day 3	Day 2
A	80 ± 20.0	20
B	80 ± 20.0	20
C	100	0
P value	0.619	NA*

*The data were not analyzed
Group A, received the mixture of the virus and the red onion extract.
Group B, received the mixture of the virus and the yellow onion extract.
Group C, received the mixture of the virus and the PBS.

The mortality rate of embryos in eggs that inoculated with the mixture of the AI virus and aqueous extract of red onion after 24 hours incubation at room temperature was only 40% on the third day post inoculation and 60% of the other embryos were hatched. The mortality rate of embryos in eggs that inoculated the mixture of the AI virus and aqueous extract of yellow onion after 24 hours incubation at room temperature was 40%, 40%, and 20% on the second day, the third day, and seventh day post inoculation respectively. The mortality rate of embryos in eggs that inoculated

the mixture of the AI virus and PBS after 24 hours incubation at room temperature was 100% on the third day post inoculation (table 4). The statistical analysis of mortality rate of treatment groups and the control group on the second and third day of incubation showed that there is no significant difference between them, and the P value was 0.4 and 0.09 respectively.

Table 4
Mortality rate of embryos in eggs after day 2, 3, and 7 of inoculation of the mixture of the AI virus and aqueous extract of red or yellow onion or PBS, after 24 hours incubation at room temperature.

Groups	Mortality rate (%)		
	Day 3	Day 2	Day 7
A	0	40 ± 24.5	0
B	20 ± 20	40 ± 24.5	20
C	0	100	0
P value	0.397	0.088	NA*

*The data were not analyzed
Group A, received the mixture of the virus and the red onion extract.
Group B, received the mixture of the virus and the yellow onion extract.
Group C, received the mixture of the virus and the PBS.

Hemagglutination titers. Mean HA titers of Avian Influenza Virus subtype H9N2 in chorioallantoic fluid (CAF) of embryonated eggs that inoculated the mixture of the virus and aqueous extract of red onion, the mixture of the virus and aqueous extract of yellow onion, and the mixture of the virus and PBS after 2 hours incubation at room temperature were 1.1, 3.5, and 4.9 respectively (table 5). Statistically, there was

Table 5
Mean hemagglutination titers (HA) of Avian Influenza Virus subtype H9N2 in chorioallantoic fluid of embryonated eggs that inoculated the mixture of the virus and PBS or aqueous extracts of red and yellow onion after 2, 8, and 24 hours incubation at room temperature.

Groups	Mortality rate (%)		
	24 hours	8 hours	2 hours
A	$1.1 \pm 0.60^{a*}$	4.1 ± 0.31^{a}	6.3 ± 0.25
B	3.5 ± 1.8^{ab}	4.8 ± 0.49^{a}	4.8 ± 0.60
C	4.9 ± 0.19^{b}	6.6 ± 0.10^{b}	6.1 ± 0.24
P value	0.013	0.000	0.082

*The data that were shown with the same letter are not significantly different.
Group A, received the mixture of the virus and the red onion extract.
Group B, received the mixture of the virus and the yellow onion extract.
Group C, received the mixture of the virus and the PBS.

a significant difference between the positive control group and group A that received the mixture of the virus and the extract of the red onion (P= 0.013). Mean HA titers of the virus in CAF of embryonated eggs that inoculated the mixture of the virus and extract of the red onion, the mixture of the virus and extract of yellow onion, and the mixture of the virus and PBS after 8 hours incubation were 4.1, 4.8, and 6.6 respectively (table 5). A statistical comparison showed that there is a significant difference between positive control group and the group that received the mixture of the virus and the extract of the red onion, and also between the two treatment groups (P= 0.000). Mean HA titers of the virus in CAF of embryonated eggs that inoculated the mixture of the virus and extract of the red onion, the mixture of the virus and extract of yellow onion, and the mixture of the virus and PBS after 24 hours incubation were 6.3, 4.8, and 6.1 respectively (table 5). Statistical analysis showed that there is no significant difference between the groups (P= 0.082).

Disscutions

AI viruses have pathological effects on chicken embryos by apoptosis and necrosis, and all of the viruses, highly pathogenic and low pathogenic AI viruses, are embryo lethal and hatching of internally contaminated eggs has not been reported [4], therefore, the mortality rate of contaminated embryos is 100%. In this study although comparison of the results of mortality rates between treatment groups and the control group indicated that there is no statistical difference between them (P>0.05), but the results clearly showed that when the mixture of the AI viruses and aqueous extract of red onion incubated 24 hours at room temperature and then inoculated to the embryonated eggs, mortality rate decreases to 40%, and 60% of the embryos survived and hatch, and about the aqueous extract of yellow onion, 20% of the embryos that had been received the mixture of the H9N2 virus and aqueous extract of yellow onion survived approximately a week, while in control group mortality rate was 100% three days post inoculation. Therefore, both extracts had antiviral effects against AI virus subtype H9N2, although the antiviral effects of red onion were more than yellow onion. Organosulfur compounds and flavonoids are important antimicrobial and antiviral compounds that extracted from onions [5, 13]. A study shows that allicin, as an organosulfur compound, has antiviral activity in addition to its antibacterial and antifungal activities [6, 14]. Flavonoid compounds including hesperetin, reduce intracellular replication of some viruses, for example, human immunodeficiency virus [8], herpes simples virus type 1, poliovirus type 1, respiratory syncytia virus, and parainfluenzavirus type 3 [9].

There is also a study that indicates onion stems can reduce the in-vitro and in-vivo infectivity of potato virus Y [10]. Therefore anti-influenza subtype H9N2 properties of the aqueous extracts of the red and yellow onions may be due to their allicin and flavonoids compounds [8]. There is a published document that shows antibacterial properties of in red onion extracts are more than yellow onion [11]. In our study, the anti-influenza virus activity of the aqueous extract of red onion was more than the aqueous extract of yellow onion. It seems the reason is due to the difference in the amount of their anti-influenza components [13].

For propagation of AI viruses, it is preferred the viruses inoculated to 9-11-day-old embryonated chicken eggs via the chorioallantoic sac [4]. In order to get higher viral titers and higher hemagglutination titers, it is necessary the stock infectious viruses diluted to 10-4 for virus propagation or to 10-9 for titration [15]; otherwise, embryos die sooner and virus titers do not increase. Therefore, if the two extracts have anti-influenza H9N2 properties, they should reduce the amount of the stock virus, so the amount of the virus propagation inside the embryonated eggs should be increased [16]. In our study, comparison of the results of mean HA titers indicated that by increasing the duration of exposure of the virus and the extracts at room temperature, the amount of virus titers in the chicken embryonated eggs increase respectively. We expected that mean HA titers of treated groups to be more than the control group [17], but mean HA titers of the control group were more than treatment groups. These results could be in addition to the direct impact due to the effect of the extracts on the propagation of the H9N2 viruses [18]. Mean HA titers of AI Virus H9N2 in the chorioallantoic fluid of embryonated eggs that inoculated the mixture of the virus and aqueous extracts of red onion after 2 and 8 hours post incubation at room temperature was less than the mixture of the virus and the aqueous extract of yellow onion. This result probably indicates that the red onion extract inhibits the propagation of the virus more than the yellow onion extract. In conclusion, according to the results of the mortality rate of the embryos and mean HA titers, it seems that both extracts especially aqueous extract of red onion not only destroys the avian influenza virus subtype H9N2, but also they decrease the propagation of the virus in chicken embryonated eggs.

Material and methods

exAvian influenza virus

Avian influenza virus subtype H9N2, A/Chicken/Iran/ZMT-101(101)/98 (H9N2), was used for this study. HA titers of the virus were 27. This virus kindly offered by the department of poultry disease, Faculty of Veterinary Medicine, University of Tehran.

Preparation of aqueous onion extract

Extracts of red and yellow onion were prepared separately. Briefly, the bulbs of red or yellow onion were washed and grated with a grater. The pulp was pressed through a clean cloth and then was centrifuged at 2000 rpm for 5 minutes at 24˚C. The extract was passed through 0.45 µm filter for sterilization.

Cytotoxicity of the extracts

A study was done to evaluate the toxic effects of the extracts. For this purpose, the onion extracts were separately diluted with sterile PBS solution at a ratio of 1/2,and 1/4. Fifteen 10-day-old embryonated chicken eggs were divided into three groups, 5 eggs/group; 0.3 ml of the undiluted extract was inoculated to the chorioallantoic sac of each egg in the first group; the other two groups received diluted extracts (1/2,and 1/4).

Antiviral effects of the aqueous extracts of the red and yellow onions.

The same method used for the evaluation of the two extracts. In order to evaluate each extract, the avian influenza virus subtype H9N2 was diluted with sterile PBS solution to 10^{-1} and then mixed with the extracts separately or PBS in 1/2 ratio. For each extract, fifteen 10-day-old embryonated chicken eggs were divided into three equal treatment groups. Fifteen other 10-day-old embryonated chicken eggs were divided into three equal positive control groups.

Group A, which includes three subgroups A1, A2, and A3, received respectively the mixture of the virus and the red onion extract after 2, 8, and 24 hours incubation at room temperature (25° C). Group B, which includes three subgroups B1, B2, and B3 received respectively the mixture of the virus and the yellow onion extract similar to group A. Group C, which includes three subgroups C1, C2, C3 received respectively the mixture of the virus and the PBS, as a positive control group, similar to the other groups (table 1). A 0.1 ml amount of the mixtures were inoculated to the chorioallantoic sacs of the eggs, and then incubated at 37° C. Mortality rate of embryos and HA titers of AI virus in the chorioallantoic fluid were recorded for evaluation.

Hemagglutination(HA) assay.

The chorioallantoic fluid of eggs whose embryos were dead on the second and third day of inoculation, harvested and titers of AI Virus subtype H9N2 of them measured as recommended by [12]. Briefly, 50 µl sterile PBS placed in every 12 wells in a microtiter plate. In the first well 50 µl of the chorioallantoic fluid of eggs added and serially diluted then, 50 µl of 0.5 % washed chicken erythrocytes added to each well, then, left at room temperature for 30-40 min. HA activity determined and expressed as reciprocal.

Statistical analysis.

One-way ANOVA and Duncan multiple range tests were used for analysis of the data. The SPSS statistics, version 22, was used for statistical analysis.

Table 1
Experimental design of the study of the antiviral effects of aqueous extracts of red and yellow onions against avian influenza virus subtype H9N2

[a] Duration of Incubation (hr)	10-day-old embryonated chicken eggs								
	Group A			Group B			Group C		
	A1	A2	A3	B1	B2	B3	C1	C2	C3
2	*			*			*		
8		*			*			*	
28			*			*			*

[a] The duration of incubation of the mixtures of the virus with PBS or aqueous extract of red or yellow onions at room temperature.
*Embryonated chicken eggs that received the mixtures after incubation at room temperature.
Group A, received the mixture of the virus and the red onion extract.
Group B, received the mixture of the virus and the yellow onion extract.
Group C, received the mixture of the virus and the PBS.

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Author Contributions

Designed the experiments: Z.R. Performed the experiments: S.A. Scientific counseling: M.V.M

Conflict of Interest

The authors declare that they have no competing interest.

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